Figure S1

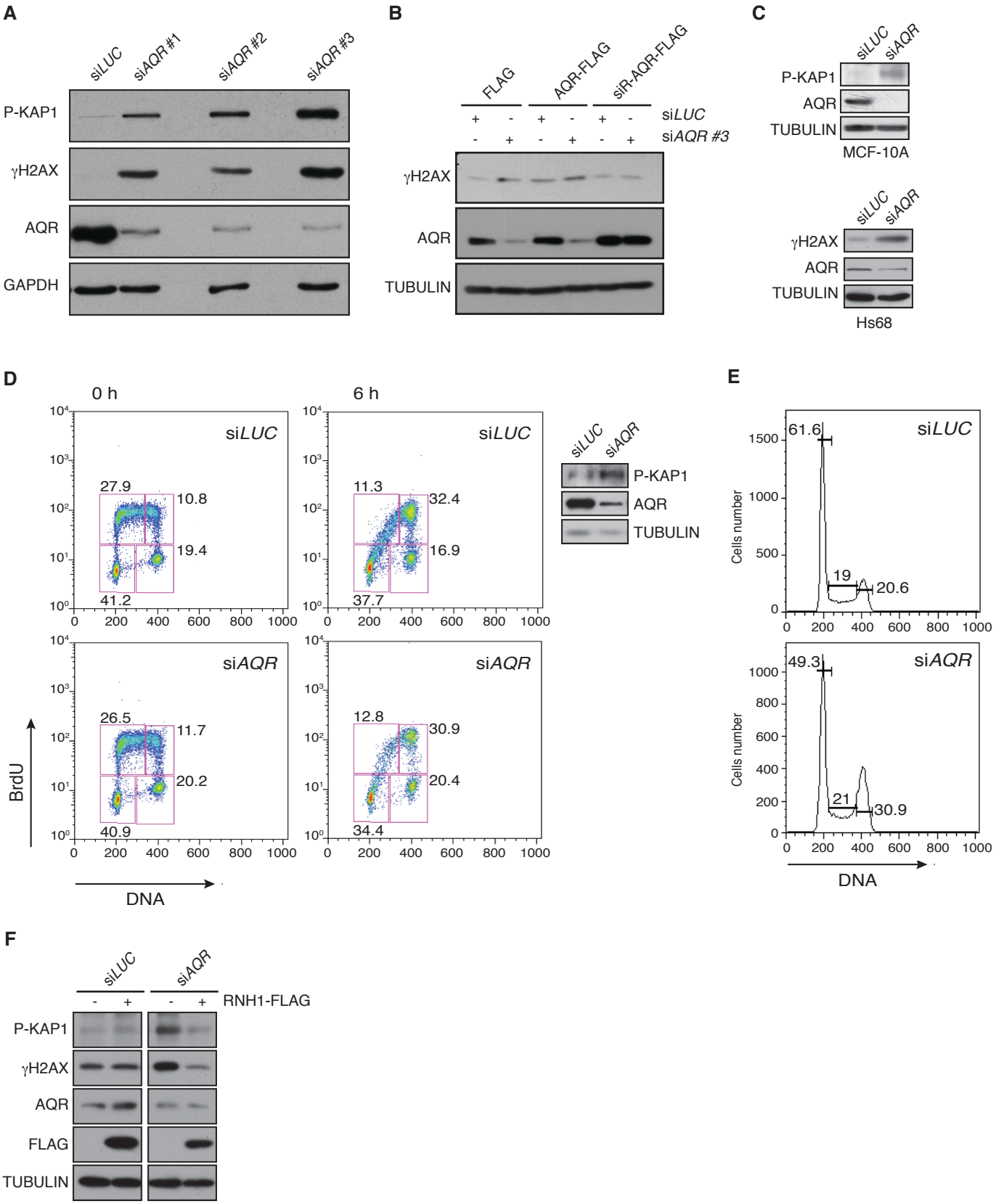
A
siLUC
siAQR #1
siAQR #2
siAQR #3
P-KAP1
γH2AX
AQR
GAPDH
B
FLAG
AQR-FLAG
siR-AQR-FLAG
siLUC
siAQR #3
γH2AX
AQR
TUBULIN
C
siLUC
siAQR
P-KAP1
AQR
TUBULIN
MCF-10A
γH2AX
AQR
TUBULIN
Hs68
D
0 h
6 h
siLUC
siAQR
27.9
10.8
19.4
41.2
11.3
32.4
16.9
37.7
26.5
11.7
20.2
40.9
12.8
30.9
20.4
34.4
BrdU
DNA
P-KAP1
AQR
TUBULIN
E
61.6
19
20.6
49.3
21
30.9
Cells number
DNA
F
siLUC
siAQR
RNH1-FLAG
P-KAP1
γH2AX
AQR
FLAG
TUBULIN

Figure S1, related to Figure 1. *AQR* knockdown leads to R-loop-induced DSBs. (A) P-KAP1 and γH2AX levels in HeLa cells transfected with si*LUC* and three different siRNAs directed against *AQR* for 72 hours. (B) γH2AX level in U2OS cells transfected with a plasmid expressing a siRNA-resistant version of AQR before transfection with si*LUC* or si*AQR* for 54 hours. (C) P-KAP1 and γH2AX levels in MCF-10A or Hs68 cells transfected with si*LUC* or si*AQR* for 54 hours. (D) FACS profiles of si*LUC* and si*AQR* cells, with DNA content marked by propidium iodide, shown on the x-axis, and BrdU incorporation shown on the y-axis. The four quadrants and corresponding values represent the percentage of cells in G1 (lower left), early/mid S (upper left), late S (upper right) and G2 (lower right). (0 h) cells were treated with siRNA for 48 hours prior to a 30 min pulse with 10 mM BrdU. (6h) cells were treated with siRNA for 48 hours, pulsed with BrdU for 30 min, and then released into nocodazole (50 ng/mL) for 6 hours. P-KAP1 level in the transfected cells used for the FACS analysis is shown. (E) FACS profiles of HeLa cells treated with si*LUC* and si*AQR* cells for 64 hours, with DNA content marked by propidium iodide shown on the x-axis, and cell content shown on the y-axis. (F) P-KAP1 and γH2AX levels in HeLa-TetON cells transfected with si*LUC* and si*AQR* 24 hours before transfection with a plasmid expressing a TET-tight inducible FLAG-tagged RNase H1 for another 48 hours. Doxycycline (100 ng/μl) was added where indicated. The gel was cropped between the si*LUC* and si*AQR* samples to remove extraneous samples.

Figure S2

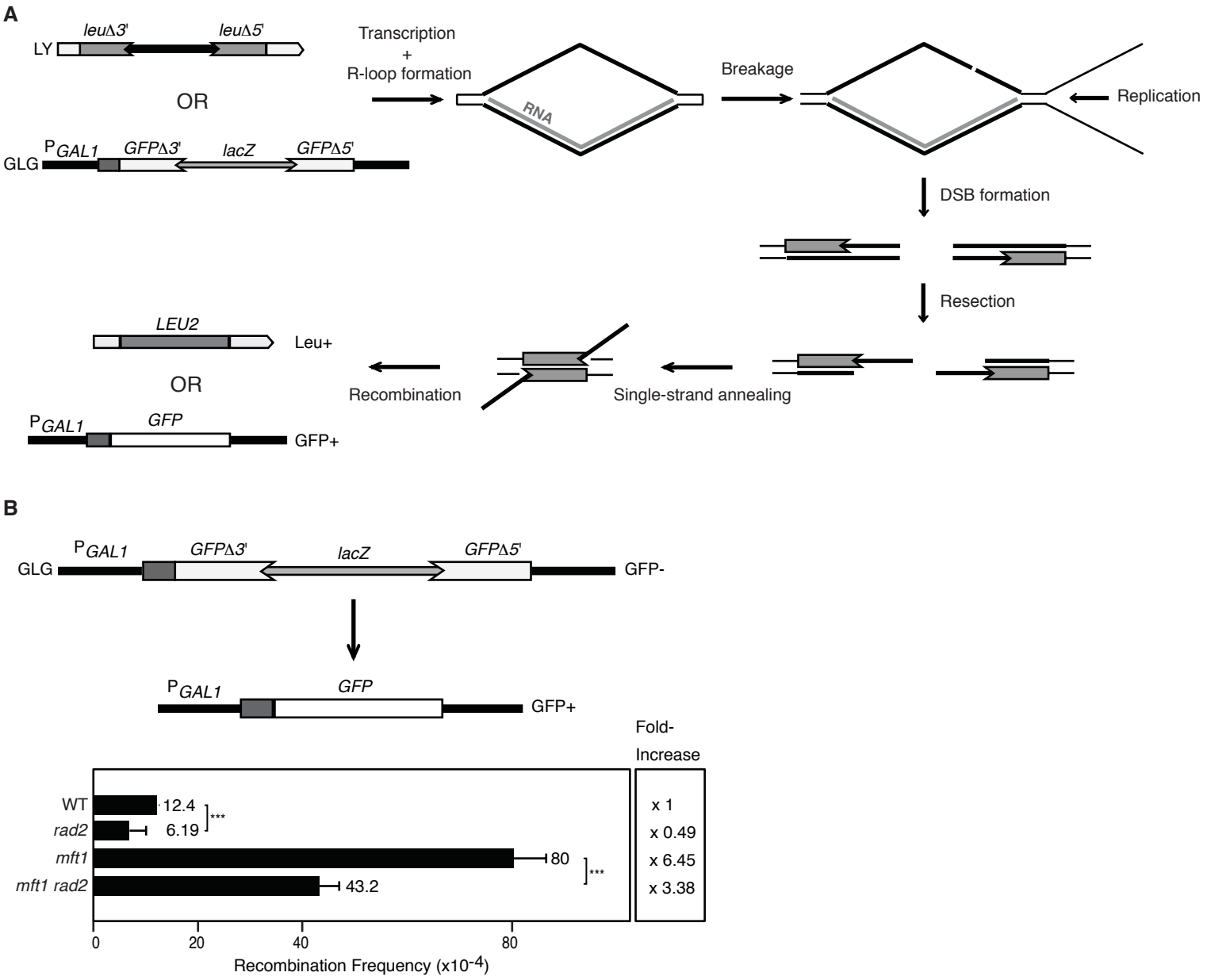
A
leuΔ3'
leuΔ5'
LY
OR
P GAL1
GFPΔ3'
lacZ
GFPΔ5'
GLG
Transcription
+
R-loop formation
RNA
Breakage
Replication
DSB formation
Resection
LEU2
Leu+
OR
P GAL1
GFP
GFP+
Recombination
Single-strand annealing
B
P GAL1
GFPΔ3'
lacZ
GFPΔ5'
GLG
GFP-
P GAL1
GFP
GFP+
Fold-
Increase
WT
rad2
mft1
mft1 rad2
12.4
6.19
80
43.2
***
***
x 1
x 0.49
x 6.45
x 3.38
0
20
40
80
Recombination Frequency (x10-4)

Figure S2, related to Figure 3 and Table S1. The processing of R-loops by the endonuclease XPG is a conserved mechanism. (A) Schematic procedure of the yeast LY and GLG recombination assays. (B) Frequencies of recombination in the GLG direct-repeat system. Each data points represent the median of 6-12 independent experiments. Error bars represent the standard error of the median (SEM, n=6-12). (***p<0.001 by Student's t-test).

Figure S3

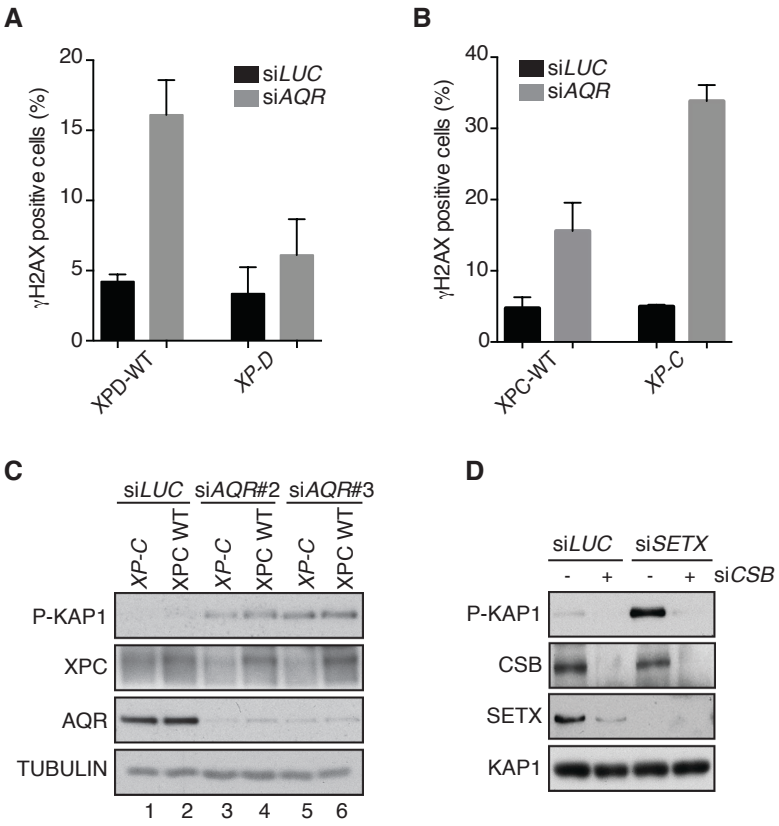
A
B
γH2AX positive cells (%)
siLUC
siAQR
XPD-WT
XP-D
XPC-WT
XP-C
C
D
siLUC
siAQR#2
siAQR#3
XP-C
XPC WT
P-KAP1
XPC
AQR
TUBULIN
1 2 3 4 5 6
siSETX
siCSB
CSB
SETX
KAP1

Figure S3, related to Figure 4. R-loop-induced DNA damage is a TC-NER-like event. (A) Quantification of percent γH2AX-positive cells in XPD-patient cell line, either complemented or not with wild-type XPD, and transfected with indicated siRNA (SEM, n=2). (B) Quantification of percent γH2AX-positive cells in XPC-patient cell line, either complemented or not with wild-type XPC, and transfected with indicated siRNA (SEM, n=3). (C) P-KAP1 level in XPC-patient cell line, either complemented or not with wild-type XPC, and transfected with indicated siRNA. (D) P-KAP1 level in HeLa cells transfected with si*CSB* or si*LUC* 24 hours before transfection with si*LUC* or si*SETX*.

Table S1, related to Figure 3 and Figure S2: Yeast strains used in this study

WM258-3D	*MAT*a *trp1-1 ura3 leu2 his3*
WM258-7B	*MATalpha trp1-1 ura3 leu2 his3*
WM258-4A	*MATalpha trp1-1ura3 leu2 his3 met15Δ0 rad2Δ::KAN mft1Δ::KAN*
WM258-8A	*MATalpha trp1-1 ura3 leu2 his3 rad2Δ::KAN mft1Δ:: KAN*
WM258-8D	*MAT*a *ura3 leu2 his3 rad2Δ::KAN mft1Δ:: KAN*
WM258-7A	*MATalpha trp1-1 ura3 leu2 his3 met15Δ0 mft1Δ:: KAN*
WM258-7B	*MAT*a *ura3 leu2 his3 mft1Δ::KAN*
WM258-7C	*MAT*a *ura3 leu2 his3 rad2Δ:: KAN*
WM258-7D	*MATalpha trp1-1 ura3 leu2 his3 met15Δ0 rad2Δ:: KAN*
WM258-10A	*MAT*a *trp1-1 ura3 leu2 his3 rad2Δ::KAN*
WM258-10C	*MATalpha ura3 leu2 his3 met15Δ0 mft1Δ::KAN*

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cell culture

All cell lines with the exception of MCF-10A were cultured in DMEM (GIBCO) supplemented with 10% FBS, 2 mM L-glutamine and penicillin/streptomycin in 5% CO2 at 37 °C. Immortalized breast epithelial MCF-10A cells were cultured in a 1:1 mixture of DMEM and F12 medium (GIBCO) supplemented with 5% horse serum, hydrocortisone (0.5 mg/ml), insulin (10 μg/ml), epidermal growth factor (20 ng/ml), cholera toxin (100 ng/ml) and penicillin-streptomycin (100 μg/ml each). To generate the HeLa-TetON-RNase H1 cell line, HeLa-TetON cells were transduced with a pLVX-Tight-Puro vector, expressing a FLAG-tagged truncated version of RNase H1. The SV40-transformed fibroblasts cells XPCS1RO (XPG-deficient), XPCS1RO + XPG-WT, XPCS1RO + XPG-E791A, XP2YO (XPF-deficient), XP2YO + XPF-WT and XP2YO + XPF-D676A were gifts from Dr. Orlando Schärer and have been described elsewhere (Ellison et al., 1998; Yagi and Takebe, 1983). The SV40-transformed XPC-deficient cell lines, XPC-deficient cell lines complemented with XPC-WT, XPD-deficient cell lines, and XPD-deficient cell lines complemented with XPD-WT (GM15983, GM16248, GM08207 and GM15877 respectively) were purchased from Coriell. The yeast strains used in this study are described in Table S1.

Antibodies, plasmids and reagents

Antibodies to phospho S139-H2AX and CHK1 (Cell signaling); AQR, ASF, SETX, CSB, phosphor S33-RPA2, phospho S824-KAP1, KAP1 (Bethyl Laboratory); H2AX, XPF, XPB, GAPDH (Abcam); XPG and CHK1 (Santa-Cruz); RPA2 (Calbiochem); XPD (Abnova); tubulin, and FLAG (Sigma) are all commercially available. The RNA-DNA antibody was purified from

the S9.6 hybridoma cell line (ATCC). Briefly, about 500 mL of hybridoma supernatant was applied to a 1 ml HiTrap Protein G HP column (GE healthcare). After washing the column with PBS 1X, the antibodies were eluted with 5 ml 100 mM Glycine pH 2.5 in 0.5 ml fractions. Fractions containing high concentrations of antibody were dialyzed in 2 L PBS 1X overnight followed by a second dialysis in 500 ml of PBS 1X/ 50% glycerol for at least five hours. The antibody was then diluted to a final concentration of 1 μg/μl and stored at -80 °C. The plasmid pcDNA3-tetO-HRH1-delta27FLAG was a gift from Xialu Li. Briefly, a FLAG tagged RNase H was inserted under the control of the TetO promoter in the pCDNA3 plasmid. The sequence encoding a truncated version of human RNase H1 was expressed from the plasmid. To induce expression of pcDNA3-tetO-HRH1-delta27FLAG, doxycycline (100 ng/μl) was added to the medium every 24 hours for 2 days. The synthetic siRNAs targeting AQR (siAQR-1, D-022214-01-05/siAQR-2, D-022214-02-05/siAQR-3, D-022214-03-05), XPG (D-006626-02-0005), CSB (D-004888-06-0005), ASF (M-018672-01-0005), SETX (custom siRNA with the following sequence GCCAGAUCGUAUACAAUUA), XPA (siXPA#1 with the following sequence GCUACUGGAGGCAUGGCU, siXPA#2 with the following sequence GGAGACGAUUGUUCAUCAA), XPC (siXPC#1, D-016040-01/ /siXPC#2, -016040-04), XPB (MU-011028-00-0002), XPD (MU-011027-01-0002) and luciferase (GL3; D-001400-01-20) were purchased from ThermoFisher. An siRNA-resistant AQR construct was generated against D-022214-03-05. si*LUC* was used as a negative control throughout the study and si*AQR#3* was used if not otherwise noted. All cell transfections were performed using 20 nM siRNA with Dharmafect 1 (ThermoFisher) following the manufacturer's protocols. Cells were harvested 64 hours after transfection of si*AQR*, si*ASF* or si*SETX* if not otherwise noted. Plasmid transfections

were performed using Fugene 6 transfection reagent (Promega) following the manufacturer's protocol.

Cell Cycle Analysis

To monitor S-phase progression, cells were pulse-labeled with 10 mM BrdU for 30 min, 48 hours after siRNA knockdown, and washed three times with PBS. After fixing samples with ice-cold 70 % ethanol, cells were permeabilized with 0.25 % Triton X-100/PBS for 15 min on ice, blocked in 2 % BSA/PBS for 15 min, and incubated in primary BrdU antibody (BD Bioscience) for 2 hours. Cells were then washed three times in PBS, incubated in AlexaFluoro-488 secondary antibody for 1 hour, and washed three times with PBS. Propidium iodide (PI; 0.1 mg/mL; Sigma) and RNase A (10 mg/mL; QIAGEN) was added to determine DNA content and cells were analyzed on a FACS Caliber (BD Bioscience) and FlowJo software.

Neutral comet assay

DNA breaks were monitored using the CometAssay Reagent Kit for Single Cell Gel Electrophoresis Assay (Trevigen, Inc) according to the manufacturer's protocol. DNA was stained with SYBR-gold (Invitrogen) and comet tail moments were calculated by counting 100 cells for each sample and analyzed with Image J (v 1.47). Tail moment (TM) reflects both the tail length (TL) and the fraction of DNA in the comet tail (TM = %DNA in tail × TL/100). The box in box-and-whiskers graphs corresponds to 25-75 percentiles. The line near the middle of the box marks the median and whiskers correspond to 10-90 percentiles. Data not included between the whiskers are plotted as outliers (dots).

γH2AX immunofluorescence

Cells were fixed with 90% ice-cold methanol for 20 min at -20°C, blocked in 2% BSA/TBS for 30 min at RT and stained with rabbit antibody to γH2AX (1:500, Cell Signaling) overnight at 4°C. Cells were then stained with DAPI and goat anti-rabbit AlexaFluoro-594-conjugated secondary antibody (1:1000, Invitrogen) for 1 hour at RT and mounted in Prolong Gold AntiFade reagent (Invitrogen). Cells were analyzed on a Zeiss Axioscope, and 40× captured images were acquired. Intensity per nucleus was calculated using Image J (v 1.47). Total intensity data was collected for the γH2AX channel. To calculate percent positive γH2AX cells, the mean and standard deviation of the total intensity were calculated for si*LUC*-transfected cells. The mean plus two standard deviations was set as the threshold for a positive cell.

Slot-blotting with S9.6 antibody

Total nucleic acids were extracted with the DNeasy Blood and Tissue kit (QIAGEN) following the manufacturer's instructions. Briefly one microgram of DNA was spotted directly on a positively charged Nylon membrane (Amersham) using a Slot-Blot apparatus (Schleicher & Schuell) in duplicate. Half of the DNA was probed with the mouse S9.6 antibody (1:1000) in 1% BSA/TBST (Tween 0.1%) overnight at 4°C after UV-crosslinking (0.12 J/m^2) and saturating the membrane with 5% milk/TBST. The other half of the DNA was denatured for 10 min in 0.5 N NaOH, 1.5 M NaCl, and neutralized for another 10 min in 1 M NaCl, 0.5 M Tris-HCl pH7.0. After UV-crosslinking (0.12 J/m^2) and saturating the membrane, total DNA was detected using the mouse single-strand DNA antibody (1:10000, Millipore). A 1:10000 dilution of goat anti-mouse HRP antibody was used as secondary antibody. Images were acquired with FluorChem

HD2 (Alpha Innotech) and quantified using Image J (v 1.47). The fold enrichment of RNA-DNA hybrids was represented with the si*LUC* samples set to one.

Immunofluorescence with S9.6 antibody

HeLa cells were transfected with the indicated siRNA for 48 hours and fixed with 100% ice-cold methanol for 5 min at -20°C. Slides were blocked in 2% BSA/PBS overnight at 4°C and incubated with S9.6 mouse (1:200) and nucleolin rabbit (1:1000, Abcam) antibodies overnight at 4°C. Slides were washed three times in 1 X PBS, and then incubated with goat anti-rabbit AlexaFluoro-488-conjugated and goat anti-mouse AlexaFluoro-594-conjugated secondary antibodies (1:1000, Invitrogen) for 1 hour at RT. Slides were washed three times in 1 X PBS and then mounted in ProLong Gold AntiFade reagent (Invitrogen). If indicated, slides were treated with human RNAse H1 (Gift for Frederic Chedin) for 36 hours at 37°C prior to overnight incubation with indicated antibodies. A Zeiss LSM500 confocal microscope with ZEN 2009 software was used and 63X captured images were acquired. Intensity per nucleus was determined using Image J (v 1.47). The Hoechst signal was used to create a mask of the nucleus. The nuclear S9.6 signal intensity was then determined by subtracting the nucleolin signal and analyzing the intensity of the remaining S9.6 signal.

Yeast LY recombination assay

Recombination frequencies in the LY direct repeat system were obtained by fluctuation tests as the median value of six independent colonies isolated from SC plates. The final frequency given for each strain and condition is the mean and standard deviation of 3-4 median values obtained from independent transformants. Recombinants were scored in SC-Leu (Luna et al., 2005).

Yeast GLG recombination assay

Transformants were grown overnight at 30°C in liquid SGal-his to allow GFP expression. The final frequency given for each strain and condition is the median recombination frequency of 6–12 different transformants (Gómez-González and Aguilera, 2007). The Start up CELLQuest software was used for data analyses and parameter optimization.

Statistical analysis

Prism (GraphPad Software) was used to perform student's t-test and non-parametric Mann-Whitney rank sum t-test. Unless specifically noted, non-parametric Mann-Whitney rank sum t-test was used to determine statistical significance. *p<0.05, ** p<0.01, ***p<0.001, and ****p<0.0001.

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